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Short Communication

Chemotactic activity of channel catfish, *Ictalurus punctatus* (Rafinesque), recombinant cyclophilin A

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Cyclophilin A (CyPA), a member of the highly conserved immunophilin superfamily, is ubiquitous (Galat 1999; Wang & Heitman 2005; Pemberton 2006). CyPA was first discovered as an intracellular receptor for the powerful immunosuppressant cyclosporine A (Handschumacher *et al.* 1984). Early studies have demonstrated that CyPA has peptidyl–prolyl *cis/trans* isomerase activity (Takahashi, Hayano & Suzuki 1989; Spitzfaden *et al.* 1992) that mediates protein folding, followed by serving as intracellular signal molecules (Brazin *et al.* 2002; Min, Fulton & Andreotti 2005; Wang & Heitman 2005). Recent studies have shown that upon stimulation, cells are able to secrete this protein, which acts as a potent chemoattractant for neutrophils, monocytes and T cells (Sherry *et al.* 1992; Xu *et al.* 1992; Billich *et al.* 1997; Allain *et al.* 2002; Yurchenko *et al.* 2002; Damsker, Bukrinsky & Constant 2007; Heine *et al.* 2011). The mechanism of leucocyte chemotaxis is involved in the direct binding of CyPA to the ectodomain of CD147 (Arora *et al.* 2005; Gwinn *et al.* 2006; Damsker *et al.* 2007). Song *et al.* (2011) further identified that residues Arg^{69}, His^{70} and Thr^{107} of CyPA are critical for binding and chemotaxis.

Cyclophilin A has also been implicated in the pathogenesis of many diseases. For example, the interaction of CyPA with HIV-1 proteins is required for successful establishment of this virus infection in the target cells (e.g. Zander *et al.* 2003; Sokolskaja, Sayah & Luban 2004; Luban 2007; Li, Kar & Sodroski 2009). Also, secreted CyPA protein acts as a proinflammatory cytokine to activate vascular endothelial cells that induce expression of adhesion molecules and apoptosis, suggesting that this protein may play a potential role in pathogenesis of atherosclerosis (Jin *et al.* 2004). Because of its role in pathogenesis, CyPA has been regarded as a disease biomarker and been an attractive target for development of therapeutic agents (e.g. Gaither *et al.* 2010; Satoh, Shimokawa & Berk 2010).

We previously observed up-regulation of CyPA transcript of channel catfish in the early stage of *Edwardsiella ictaluri* infection (Yeh & Klesius, unpubl. data). Subsequently, we identified and characterized channel catfish CyPA transcript, which contains one open reading frame potentially encoding a 164 amino acid peptide (Yeh & Klesius 2008). In this communication, we report expression and purification of channel catfish CyPA from an *Escherichia coli* expression system and determine its chemotactic activity.

Channel catfish, *Ictalurus punctatus* (Rafinesque), obtained from the USDA ARS Catfish Genetics Research Unit, Stoneville, MS, were used in the experiments. Fish were maintained according to the procedures established at the USDA ARS Aquatic Animal Health Research Unit (AAHRU), Auburn, AL (Jenkins & Klesius 1998; Klesius, Evans & Shoemaker 2007). Specific pathogens of channel catfish were monitored by various methods, such as loop-mediated isothermal amplification (Yeh, Shoemaker & Klesius 2005, 2006). All catfish were clinically healthy. The protocol for animal usage in this study was approved by the Institutional Animal Care and Use Committee, AAHRU, Auburn, AL.

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Peritoneal macrophages were collected using the method established by Klesius & Sealey (1996) and Jenkins & Klesius (1998). Channel catfish with a mean weight of 23.3 ± 0.9 g and length of 12.8 ± 0.3 cm were injected with 100 μL each of squalene (Sigma Chemical Co.,) and held for 9 days prior to cell collection. Three individual fish were anaesthetized in 125 mg L^{-1} tricaine methanesulfonate (Tricaine-S, Western Chemical) and used to collect cells via peritoneal lavage with ice-cold phosphate-buffered saline. After collection, cells were washed in Hank's balanced salt solution without Mg^{+2} and Ca^{+2}. After washing, cells were suspended into 1–2 mL of RPMI-1640 medium (Invitrogen), supplemented with 5% heat-inactivated foetal horse serum (HS) and counted with a haemocytometer.

Total RNA was isolated from anterior kidney, and cDNA synthesis was performed as previously described (Yeh & Klesius 2008) using commercial kits.

The full-length channel catfish CyPA cDNA was PCR-amplified using the following primers: (1) 5′- CTCggatccATGAGCAAGCCTAAAGTGTT TTTTGAC-3′ (forward) and (2) 5′-GAGctcgagTT ATTAAAGTTGGCCGCAGTCAGCGATGACT AT-3′ (reverse). Two restriction sites (*Bam*HI and *Xho*I) in lowercase were added in the forward and reverse primers, respectively. PCR reactions in 50 μL contained the following reagents (in final concentrations): 1× PCR buffer (TaKaRa Bio USA), 200 μM of each dNTP (TaKaRa Bio USA), 5 mM $MgCl_2$ (Invitrogen), 0.3 μM of each primers (synthesized by Sigma-Genosys), 1.25 U *Ex Taq* HS DNA polymerase (TaKaRa Bio USA) and cDNA template. The amplification was carried out initially at 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 s, 62 °C for 1 min and 72 °C for 2 min. The final extension was performed at 72 °C for 10 min. The reaction without addition of cDNA template served as a negative control. The PCR products were purified by agarose gel electrophoresis, ligated into a pSC-A vector (Stratagene), transformed and replicated in *Escherichia coli* (Stratagene). The CyPA-containing plasmids in *Escherichia coli* were purified by a Plasmid Purification Kit (Qiagen), digested with *Bam*HI and *Xho*I restriction enzymes (Promega), ligated into the corresponding restriction enzyme-digested pET30a bacterial expression vector (Novagen) and transformed into *Escherichia coli* BL21 (DE3) (Novagen).

Positive clones of *Escherichia coli* BL21 (DE3) were grown in LB medium containing 30 μg mL^{-1} kanamycin at 35 °C to an $OD_{600\ nm}$ at 0.4 and induced for protein expression with 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) at 25 °C overnight. Cells were harvested by centrifugation, washed once and lyzed by sonication. Soluble and insoluble fractions were separated by centrifugation and examined by SDS-PAGE to determine which fraction contained the expressed protein. The expressed protein that was recovered in insoluble fraction was purified in the presence of 6 M urea using a PrepEase His-Tagged Protein Purification Kit (USB Corporation) according to the manufacturer's protocol. The purified protein was dialysed against a series of decreasing concentrations of urea in the PBS buffer and finally in 35 mM HEPES buffer (pH 8.0) at 4 °C. The recombinant protein was filter sterilized and stored at −20 °C.

SDS-PAGE was carried out by a Criterion precast gel system (Bio-Rad Laboratories). Samples were solubilized in 2X Laemmli sample buffer (Bio-Rad Laboratories) and boiled for 3 min, and separated in 15% precast Tris-HCl polyacrylamide gels (Bio-Rad Laboratories). After separation, the proteins were stained with Coomassie Brilliant Blue R250 in a staining solution (45% methanol, 10% acetic acid and 0.5% Coomassie brilliant blue R-250) for 90 min at room temperature, followed by destaining with a destaining solution (20% methanol and 10% acetic acid). Images were documented by a KODAK Gel Logic 440 Imaging System and processed by AlphaView SA software.

The recombinant channel catfish CyPA was excised from SDS-PAGE and was sent to Alphalyse North America for protein identification by MALDI-TOF/TOF mass spectrometry. The MS spectral data were searched against the protein databases by the MASCOT software (v. 2.2.03) (Matrix Science).

Chemotaxis was determined using blind-well chemotactic chambers (Corning CoStar,), with the two compartments separated by an 8 μm pore size polycarbonate membrane (Nucleopore) as describe by Boyden (1962) and adapted by Klesius & Sealey (1996). Briefly, the lower compartment was filled with 0–28 μg of purified recombinant CyPA (rCyPA), and the difference made up to 200 μL with RPMI-1640 plus 1% HS. The solution for the negative control (the concentration of rCyPA at 0 μg per 200 μL point in Fig. 3) contained 50:50

of RPMI-1640 plus 1% HS and filtered solution for eluting the purified rCyPA protein. The filters were treated with RPMI-1640 plus 1% HS prior to placing in the chamber. After insertion of the membrane, a 100-μL aliquot of peritoneal macrophages ($1.5–1.7 \times 10^6$ cells mL^{-1}) suspended in RPMI-1640 plus 1% HS were seeded into the upper compartment. Each concentration was completed in duplicate chambers, and the response to rCyPA was determined from peritoneal macrophages collected from three individual fish. The chambers were incubated at 25 °C on a horizontal platform shaker at 100 rpm. After 90-min incubation, the filters were removed, inverted and mounted on cleaned slides. The slides were stained with Protocol Hema 3 stain (Fisher Scientific Co.), in a manner similar to Klesius & Sealey (1996). Enumeration of migrating macrophages was achieved by counting five fields of view on the bottom surface of the filters with a light microscope at 400× magnification.

After the channel catfish CyPA was characterized at the cDNA level (Yeh & Klesius 2008), we further evaluated the roles of the CyPA protein in teleost fish immunological functions. First, the full-length cDNA of channel catfish CyPA was PCR-amplified, cloned and transformed into the competent *E. coli* BL21 (DE3) cells. As seen in Fig. 1, the SDS-PAGE analysis of the expressed clone showed a major protein band with an estimated molecular weight of 19.9 kDa that is higher than the predicted molecular mass from the deduced amino acid sequence. This difference between apparent molecular mass determined by SDS-PAGE and calculated molecular mass is partly due to inclusion of a six-His tag sequence. In addition, this difference has been reported in many recombinant proteins (Goshima *et al.* 2008). This discrepancy may also be due to the degree of binding of the recombinant proteins to the detergent SDS (Robinson & Tanford 1975; Takano *et al.* 1988; Goshima *et al.* 2008). To confirm that this recombinant protein was channel catfish CyPA, the 19.9 kDa band from the SDS-PAGE gel was excised and subjected to MALDI-TOF mass spectrometric analysis. As seen in Fig. 2, peptide fragments of channel catfish CyPA were identified by MALDI-TOF mass spectrometry with about 51% coverage of the sequence. All peptides were matched to the deduced channel catfish CyPA amino acid sequence, suggesting that the 19.9 kDa recombinant protein is the channel catfish CyPA peptide. Because the channel catfish CyPA was fused to a tag peptide during expression vector construction, the His tag peptide was also detected by MALDI-TOF mass spectrometry. Together, the channel catfish CyPA was expressed and confirmed.

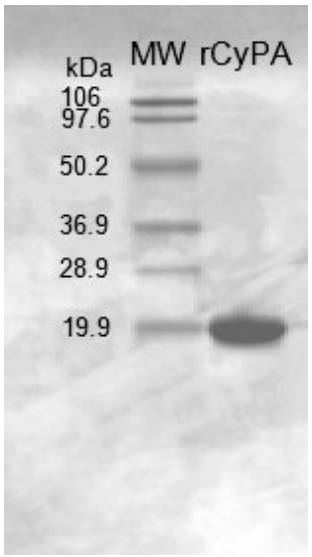

Figure 1 SDS-PAGE analysis of channel catfish cyclophilin A (rCyPA) expressed and purified from *E. coli* BL21 (DE3) cells. Sample was solubilized in a 2× Laemmli sample buffer and separated on a 15% gel (Bio-Rad). After extensive wash, rCyPA was eluted with 250 mM imidazole (lane rCyPA). MW, molecular mass standards (Bio-Rad), are indicated at the left column.

To purify this CyPA recombinant protein from the *E. coli* expression system, a PrepEase His-tagged Protein Purification Kit was used. In our preliminary result, we found the recombinant protein was neither secreted into supernatant nor soluble in the cytoplasm. Thus, a final concentration of 6 M urea was added to solubilize the inclusion bodies, followed by binding to nickel–iminodiacetic acid beads in a column and eluting with 250 mM of imidazole. As shown in Fig. 1, the rCyPA protein was purified almost to homogeneity analysed by SDS-PAGE.

Several studies in fish and shellfish have demonstrated that the CyPA transcript was up-regulated in the early stage of bacterial infection (Yeh & Klesius 2008; Qiu *et al.* 2009; Song *et al.* 2009). These observations prompted us to hypothesize that the CyPA protein plays a critical role in the teleost innate immune system. In this study, rCyPA induced migration of channel catfish peritoneal macrophages *in vitro*. As depicted in Fig. 3, peritoneal macrophages from channel catfish migrated in response to rCyPA in a dose–response

MSKPKVFFDITIDGKSAGRIVIELRA**DVVPKTAENFRALCTGEKGFGYKGSGFHRVIPGF**
MCQGGDFTNHN**GTGGKSIYGNKFADENFTLKHTGPGIMSMANAGPNT**NGSPFFICTEKTS
WLDGKH**VVFGSVVDGMDVV**RVVEGRGSSSGKCFAKIVIADCGQL

Figure 2 Analysis of the recombinant channel catfish cyclophilin A protein by MS peptide mapping and sequencing analysis. The protein band stained with Coomassie blue was excised from the SDS-PAGE and digested with trypsin. The resulting fragments were concentrated and eluted onto an anchor chip target for analysis on a Bruker Autoflex III MALDI-TOF/TOF instrument. The MASCOT score was 193, and the mass spectrometric spectra of the peptide fragments with about 51% (*e* value 8.9×10^{-14}) coverage of the sequence were identified as channel catfish cyclophilin A protein (GenBank accession no.: NP_001187167) after search against the protein databases by the MASCOT software. The matched peptides are indicated in bold and underlined.

manner and reached a plateau at the higher concentrations of rCyPA. This finding is in agreement with observations that CyPA induces leucocyte migration in the mammalian counterparts (Sherry *et al.* 1992; Xu *et al.* 1992; Billich *et al.* 1997; Allain *et al.* 2002; Yurchenko *et al.* 2002; Damsker *et al.* 2007). High homology of CyPA among species may explain why the channel catfish CyPA protein functions similarly. The key residues of CyPA involved in chemotaxis in mammals have been mapped to His^{70}, Thr^{107} and Arg^{69} (Song *et al.* 2011). By comparison of the CyPA amino acid sequences, CyPA of channel catfish conserves His^{70}, Thr^{107}, but not Arg^{69} (Yeh & Klesius 2008). In teleost fish, Asn^{69} is conserved in all fish examined. Whether the Asn^{69} or Arg^{69} residue is critical for chemotaxis in fish remains to be determined. Our long-term objective in the near post-genome era for teleosts is to develop a monitor system for assessing aquatic animal health in intensive aquaculture. Therefore, identification of novel factor(s) in the fish sera and/or body fluid during inflammatory processes as targets for monitoring the fish health status is our primary goal. As mentioned previously, high levels of the CyPA transcript have been observed in the early stage of bacterial infection so that a rationale for further evaluation of *in vitro* function of the channel catfish CyPA protein on chemotaxis was examined. Our *in vitro* result in this study clearly indicates that the rCyPA protein is a chemoattractant and has the capacity to induced peritoneal macrophage migration. The significance of our current study is to provide a fundamental framework to further study: (i) whether the CyPA induces other fish leucocyte migration *in vitro* and (ii) whether our finding *in vitro* is also valid in *in vivo* recruitment of

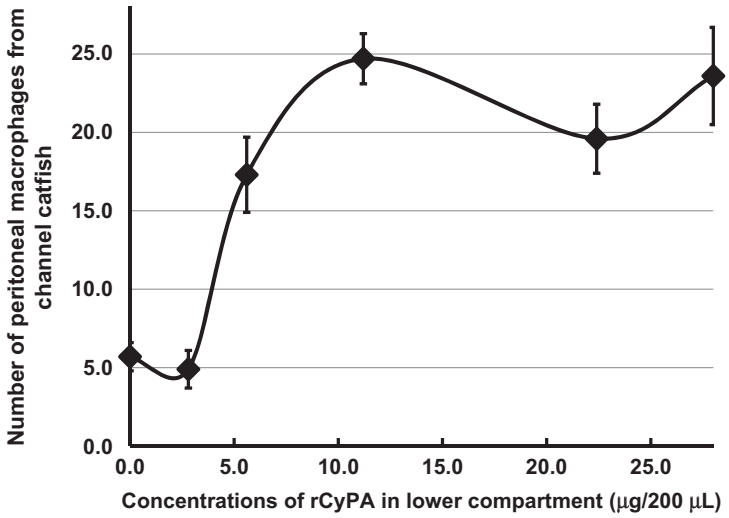

Figure 3 *In vitro* chemotactic response of peritoneal macrophages from channel catfish to recombinant cyclophilin A (rCyPA). The means with standard errors are the results of three individual catfish peritoneal macrophages assayed in duplicated chambers following counting of five individual fields at 400×.

leucocytes into tissues during inflammatory processes, where a battery of cytokines and chemokines is involved. Because of the lack of immunological reagents for aquatic animals, it is anticipated that this recombinant CyPA protein will be very useful for future development of monoclonal and polyclonal antibodies to investigate this protein in the teleost immune and pathogenic processes.

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